JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2009, p. 796–799 Vol. 47, No. 3
0095-1137/09/$08.00+0 doi:10.1128/JCM.01228-08

Characterization of a Canadian Mink H3N2 Influenza A Virus Isolate Genetically Related to Triple Reassortant Swine Influenza Virus▽

Carl A. Gagnon,[1,2]* Grant Spearman,[3] Andre Hamel,[4] Dale L. Godson,[5] Audrey Fortin,[2] Guy Fontaine,[1] and Donald Tremblay[1]

Service de Diagnostic,[1] Faculté de Médecine Vétérinaire,[2] Université de Montréal, St. Hyacinthe, Quebec, Canada; Nova Scotia Department of Agriculture, Truro, Nova Scotia, Canada[3]; Veterinary Diagnostic Services Manitoba Agriculture, Food and Rural Initiatives, Winnipeg, Manitoba, Canada[4]; and Prairie Diagnostic Services, Western College of Veterinary Medicine, Saskatoon, Saskatchewan, Canada[5]

Received 28 June 2008/Returned for modification 3 November 2008/Accepted 22 December 2008

In 2007, an H3N2 influenza A virus was isolated from Canadian mink. This virus was found to be phylogenetically related to a triple reassortant influenza virus which emerged in Canadian swine in 2005, but it is antigenically distinct. The transmission of the virus from swine to mink seems to have occurred following the feeding of animals with a ration composed of uncooked meat by-products of swine obtained from slaughterhouse facilities. Serological analyses suggest that the mink influenza virus does not circulate in the swine population. Presently, the prevalence of influenza virus in Canadian farmed and wild mink populations is unknown. The natural occurrence of influenza virus infection in mink with the presence of clinical signs is a rare event that deserves to be reported.

In 1998, an H3N2 triple reassortant influenza virus emerged in the U.S. swine population (10, 19, 21). Its eight RNA viral genes were identified to be (i) of human virus lineage for the hemagglutinin (HA), neuraminidase, and RNA polymerase (PB1) genes; (ii) of classical swine virus lineage for the nucleoprotein (NP), matrix (M), and nonstructural (NS) genes; and (iii) of North American avian virus lineage for the RNA polymerase (PA and PB2) genes. Unfortunately, the H3N2 triple reassortant subtype of influenza virus emerged in the Canadian swine and turkey populations in 2005 and is now widespread across the country (17). The H3N2 subtype of swine influenza virus (SIV) had not crossed the United States-Canadian border prior to 2005, which explains why it had not been reported to occur in the Canadian swine population since the mid-1990s (17). Today, the SIV subtypes that are mostly found in Canada are usually H1N1 and H3N2 (8, 17). Other subtypes, such has H1N2, H3N3, and H4N6, were recently reported to be present in the Canadian swine population and are sporadically found (8, 9, 11).

Mink have previously been known to be susceptible to influenza virus infection (14–16, 20). However, an influenza virus outbreak in a mink population associated with the development of clinical signs in infected animals is a rare event, and it has been reported only on a few occasions, such as the 1984 Sweden outbreak caused by an avian H10N4 influenza virus (12). Therefore, it is believed that most of the natural influenza virus infections in mink are asymptomatic, with the consequence that no specific influenza virus subtype has been known to circulate in the mink population. It is noteworthy that under experimental conditions, it was demonstrated that several subtypes of avian, human, and equine influenza viruses were able to infect mink (5, 6, 14, 15, 20). Avian and swine are currently considered to be the most important species epidemiologically involved in mechanisms that possess highly pandemic potential for the human population, such as reassortment. It is very important to do epidemiological surveillance of influenza virus not only within common susceptible animals, like avian and swine populations, but also for all other species where intensive production and high geographic densities of animals may favor the appearance of new influenza virus isolates. Herein, the genomic and antigenic characterization of a H3N2 triple reassortant influenza virus isolated from Canadian mink is reported.

In 2006, mink ranches in the province of Nova Scotia, Canada, were experiencing increases in respiratory problems, such as pneumonia, with various rates of mortality. The mortality rates were higher in ranches where other pathogens, like Aleutian disease virus of mink, were also present. Two animals, 6 months of age, experiencing clinical signs, such as dry cough, were necropsied. No macroscopic lung lesion was observed. Histopathological examination revealed the presence of mild interstitial pneumonia and mild to moderate bronchiolitis. Bacteriological cultures of the lungs were negative. A PCR diagnostic test using a specific primer set previously described by others (18) was performed on lung tissues. The result was positive for the presence of influenza A virus. Afterwards, another PCR diagnostic test (3), able to differentiate between H1, H3, N1, and N2 subtypes, was performed. The result was positive for the H3N2 subtype. Furthermore, results for immunohistochemistry using a polyclonal anti-NP antibody (National Institute of Allergy and Infectious Disease, Bethesda, MD) were positive in lungs. Unfortunately, attempts to isolate the virus from lung homogenates following inoculation of Madin-Darby canine kidney cells or embryonated chicken eggs were unsuccessful. In 2007, lung samples of mink from the same geographical region were taken for an influenza A virus

* Corresponding author. Mailing address: Faculté de médecine vétérinaire, Université de Montréal, 3200 rue Sicotte, St. Hyacinthe, Quebec, Canada J2S 7C6. Phone: (450) 773-8521, ext. 18681. Fax: (450) 778-8113. E-mail: carl.a.gagnon@umontreal.ca.

▽ Published ahead of print on 30 December 2008.

surveillance program. Some samples were positive for influenza A virus by PCR. Then, attempts to isolate the virus were made. In one case, an influenza A virus (A/Mink/Nova Scotia/1055488/07, abbreviated Mk/NS/1055488/07) was isolated only in Madin-Darby canine kidney cells. The presence of the virus was confirmed by PCR (18) and electron microscopy (13). Surprisingly, no viruses were isolated in embryonated chicken eggs. The lung sample from which the virus was isolated originated from an animal that was raised in a Nova Scotia mink ranch and never experienced any respiratory problems.

The viral RNA was isolated from the MkNS/1055488/07 virus by using a commercial kit (QIAamp viral RNA minikit; Qiagen, Mississauga, Ontario). The full lengths of the viral RNA segments were amplified by reverse transcription-PCR, the PCR products were purified (QIAquick PCR purification kit; Qiagen), and both strands of the purified DNA PCR products were sequenced by using the same primer sets (7, 10) used in the reverse transcription-PCR, with standard automated sequencing methods (Faculté de médecine vétérinaire Sequencing Laboratory, BigDye Terminator sequencer version 3.1 [ABI 310]; Applied Biosystems, Foster City, CA). The resulting sequences were compared with sequences of SIV reference strains available in the Diagnostic Veterinary Virology Laboratory of the Université de Montréal (such as a virus isolated in the province of Quebec during the 2005 H3N2 Canadian SIV outbreak, A/Swine/Quebec/4001/05 [Sw/Qc/4001/05]) and in the GenBank database. Software (BioEdit sequence alignment editor version 7.0.9; Ibis Therapeutics, Carlsbad, CA) using the CLUSTAL W alignment method was utilized, and an unrooted phylogenic tree of the HA gene was constructed by using the distance-based neighbor-joining method. Bootstrap values were calculated for 1,000 repeats of the alignment. The nucleotide identities between Mk/NS/1055488/07 and the most recent 2005 Canadian swine (A/Swine/Manitoba/12707/05, A/Swine/Alberta/14722/05, A/Swine/British Columbia/28103/05, A/Swine/Ontario/33853/05 [abbreviated Sw/Mn/12707/05, Sw/Ab/14722/05, Sw/BC/28103/05, and Sw/On/33853/05, respectively], and Sw/Qc/4001/05), turkey (A/Turkey/Ontario/31232/05, abbreviated Tk/On/31232/05), and human (A/Ontario/RV1273/05, abbreviated On/RV1273/05) triple reassortant influenza virus isolates (17) were determined. They were established to range from 97.5% to 98.4% for HA, 98.0% to 98.9% for neuraminidase, 98.3% to 99.1% for PB1, 98.1% to 99.0% for PB2, 98.7% to 99.3% for PA, 99.2% to 99.4% for NP, 99.2% to 99.3% for M, and 99.0% to 99.1% for NS genes (data not shown). Following the use of the classification adopted by Olsen and his collaborators (17), an HA nucleotide unrooted phylogenetic tree was constructed using reference strains of each cluster and all previously reported 2005 H3N2 Canadian reference strains (Fig. 1). As illustrated in Fig. 1, the Mk/NS/1055488/07 isolate was classified within the same cluster (IV) as all 2005 Canadian H3N2 SIV isolates previously classified (17). Also, the genetic relatedness of Mk/NS/1055488/07 to older Canadian H3N2 strains, such as A/Swine/Quebec/150/90 (Sw/Qc/150/90) (1, 2), was much lower than that of recent H3N2 Canadian strains (Fig. 1). When a BLAST analysis (using http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed in June 2008 with all the viral RNA genome segments of Mk/NS/1055488/07, the recent triple reassortant Canadian influenza viruses reported by Olsen and collaborators (17) were genetically the most closely related to the mink H3N2 influenza virus (data not shown).

Antigenic comparisons of the Mk/NS/1055488/07 isolate with (i) the Sw/Qc/4001/05 Quebec reference strain that had previously been classified in the same cluster (IV) (Fig. 1), (ii) a reference strain of cluster II (A/Swine/Iowa/8548-1/98, abbreviated Sw/Ia/8548-1/98), and (iii) an older Quebec H3N2 strain (Sw/Qc/150/90) were done by using a hemagglutination inhibition (HI) assay. The sera used in the HI assay were obtained following immunization of chicks with formalin-inactivated viruses (ck/150/90 and ck/Ia/98) or from naturally infected pigs (sw/4001/05). The HI assay was performed as previously described, with minor modifications (4). The H3N2 influenza-specific sera possessed various titers of HI antibodies against all H3N2 influenza strains, but the HI antibody titer of each serum sample was highest for its homologous strain (Table 1). The most striking result was that all H3N2 influenza-specific reference sera reacted poorly against the mink influenza virus isolate (Table 1). As an example, the pig serum sample specific for the most recent influenza virus isolated in Quebec (sw/4001/05) had an HI antibody titer of 1,280 for its homologous strain (Sw/Qc/4001/05), compared to an HI antibody titer of 80 for Mk/NS/1055488/07 (Table 1). Similarly, the HI antibody titers of ck/150/90 and ck/Ia/98 sera were 1,280 and 160, respectively, for their homologous strains and were only 80 and 20, respectively, for Mk/NS/1055488/07. These latest results clearly indicate that the Mk/NS/1055488/07 isolate is antigenically distinct from other strains and also distinct from a strain previously classified in the same genomic cluster (IV) as the Sw/Qc/4001/05 strain (Fig. 1 and Table 1).

Unfortunately, the spread of influenza virus in the mink population could not be evaluated, because there were not enough samples submitted to the diagnostic laboratory. On the other hand, thousands of swine samples are submitted each year to the Diagnostic Veterinary Virology Laboratory. Consequently, the spread of the mink influenza virus in the swine population was evaluated. Thus, the HI antibody titers for the Sw/Qc/4001/05 and Mk/NS/1055488/07 strains in 100 pig sera obtained between July 2007 and March 2008 were evaluated. All sera were obtained from pigs housed in farms in Quebec, Canada, and each serum sample was obtained from a different farm. To establish if the HI serum antibody titers obtained were significantly different, the nonparametric Wilcoxon matched-pair *t* test was applied using GraphPad Prism version 4 software. Twenty-three percent of the tested sera were HI antibody negative for both viruses (data not shown). The mean HI values of HI antibody-positive sera were 292.3 and 79.9 for Sw/Qc/4001/05 and Mk/NS/1055488/07, respectively (Fig. 2A). The difference between Sw/Qc/4001/05 and Mk/NS/1055488/07 HI antibody titers was significant, with a P value of <0.0001 (Fig. 2A). Furthermore, no pig serum sample had a higher HI antibody titer for Mk/NS/1055488/07 than for Sw/Qc/4001/05, with the exception of two sera that had the same HI antibody titers for both strains (Fig. 2B), suggesting that this particular mink influenza virus isolate does not seem to circulate in the swine population.

The present article reports a rare event, which is the natural occurrence of influenza virus infection in mink with the presence of clinical signs (5). The mink influenza virus isolate was identified to be genetically related to the H3N2 triple reassor-

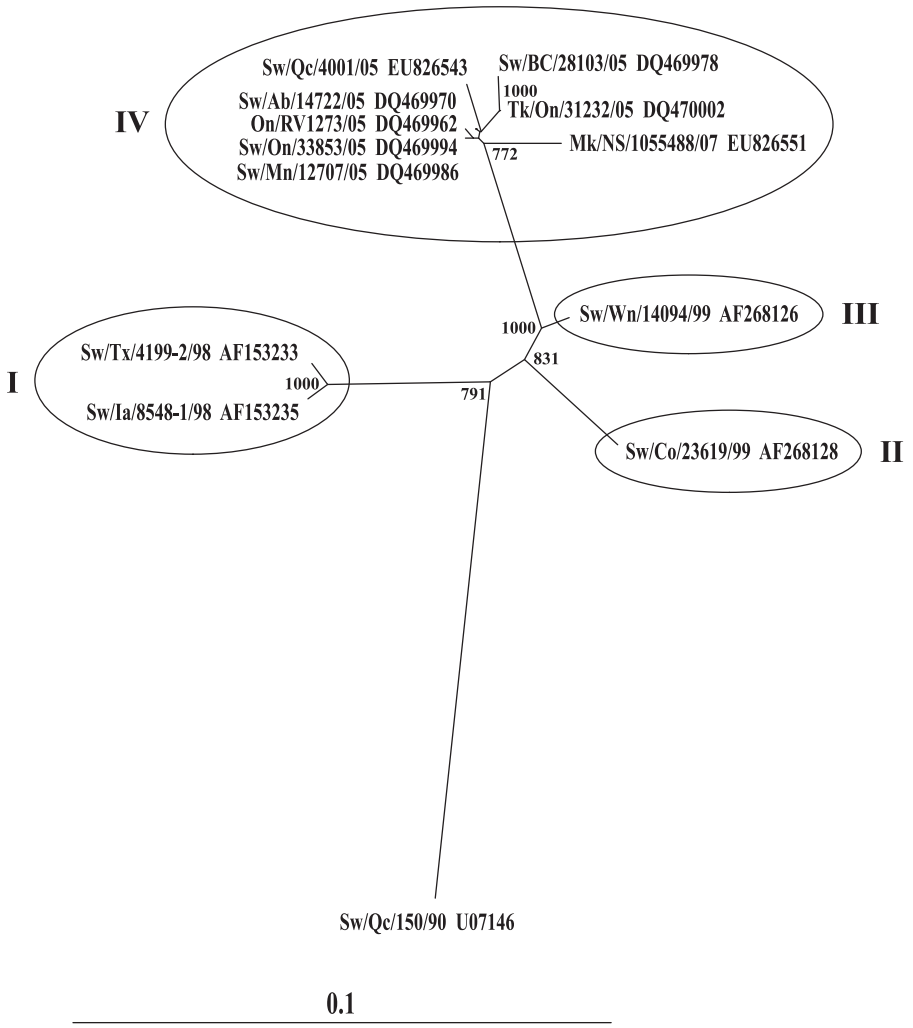

FIG. 1. Phylogenetic tree of the HA gene nucleotide sequences of recent H3N2 triple reassortant Canadian influenza virus isolates. The GenBank accession number of each strain is indicated on the right, next to the name of the strain. The strains have been classified in four distinct clusters (I to IV), as previously described (17). Bootstrap values are indicated, except for cluster IV, where some are omitted for clarity. The horizontal scale bar indicates the distances between strains; a 0.1 distance means that the strains possess 90% nucleotide identity.

tant SIV that emerged in Canadian swine and turkey populations in 2005 (Fig. 1) (17). It is difficult to establish if the virulence of the disease found in mink is different from that found in H3N2 influenza virus-infected swine and turkey populations. Occasionally, the disease in mink seemed to be more severe, but it was exacerbated by the presence of other pathogens, such as mink parvovirus. If we assume that the origin of the virus in the mink population is swine, then what is the link between the two species? The only hypothesis that could answer this question involves the type and origin of food that was given during the 2006 outbreak in mink. All of the mink on the mink ranches where the influenzalike syndrome was observed were fed a ration prepared in large batches at the same location, referred to as a kitchen. The ration was composed of uncooked meat by-products of swine, beef, and poultry obtained from slaughterhouse facilities. The swine by-product included ground lung purchased in large frozen blocks from areas of the country where swine influenza H3N2 is known to be prevalent. Swine influenza had not been detected in Nova Scotia's swine in surveillance testing prior to the mink influenza outbreak. To our knowledge, no other animal species except mink were fed with this ration of uncooked meat by-products of swine. Even when the mink influenza virus was isolated from asymptomatic animals, the animals of that ranch

TABLE 1. Antigenic reactivities of mink influenza virus isolates with specific reference antisera as determined by the HI assay

Serum sample[a]	HI antibody titer for indicated strain			
	Sw/Qc/150/90	Sw/Ia/8548–1/98	Sw/Qc/4001/05	Mk/NS/1055488/07
ck/150/90	1,280	40	640	80
ck/Ia/98	<10	160	160	20
sw/4001/05	10	40	1,280	80

[a] ck/150/90 and ck/Ia/98 are sera that were obtained following immunization of chicks with formalin-inactivated viruses (Sw/Qc/150/90 and Sw/Ia/8548–1/98, respectively); sw/4001/05 is a serum sample that was obtained from pigs naturally infected with Sw/Qc/4001/05.

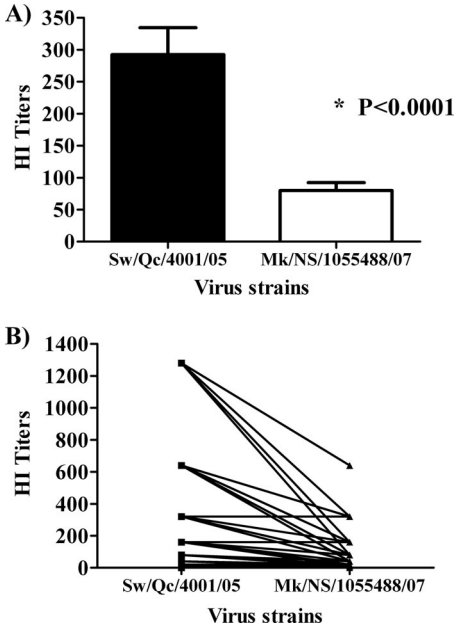

FIG. 2. HI antibody titers of swine sera for a 2005 H3N2 Canadian SIV isolate and mink influenza virus. The HI antibody titers of swine sera for the Quebec SIV reference strain (Sw/Qc/4001/05) were compared to the HI antibody titers for the mink influenza virus isolate (Mk/NS/1055488/07). (A) The mean HI antibody titers of the sera that tested positive are represented along with the standard errors of the means. (B) The absolute HI antibody titers of each serum sample for both viruses are illustrated.

were fed in 2006 with the same batch of feed that was related to the appearance of the disease in several other Nova Scotia ranches. Depending on the level of biosecurity of each ranch, it is possible that the mink influenza virus could be transmitted to wild mink or other wild species, but at this time, that is only a speculation. In the future, the mink industry should be careful with respect to their management procedures, including the use of slaughterhouse uncooked meat by-products, to avoid emerging diseases in mink which might favor the appearance of pathogens, such as influenza virus, that could have a harmful impact on public health safety. Unfortunately, the prevalence of influenza virus in Nova Scotia's farmed and wild mink populations is unknown, and it would be interesting to conduct new epidemiological studies to follow the genetic evolution and distribution of influenza virus in their populations.

Nucleotide sequence accession numbers. The GenBank accession numbers assigned to the gene sequences determined for this report are as follows: EU826543 to EU826550 for Sw/Qc/4001/05 and EU826551 to EU826558 for Mk/NS/1055488/07.

This work was supported by the National Sciences and Engineering Research Council of Canada (NSERC) discovery grant (to C.A.G.) and the diagnostic service of the Faculté de médecine vétérinaire of the Université de Montréal.

We are grateful to Cynthia M. Guilbert and Rémi Gagnon for critically reviewing the manuscript. We are also grateful to Brigitte Bousquet and Denis St. Martin for their technical assistance. We also thank Josée Harel for providing access to her laboratory equipment.

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